MEIOTIC MATURATION AND EARLY DEVELOPMENT IN THE MARINE BIVALVE HIATELLA FLACCIDA

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MEIOTIC MATURATION AND EARLY DEVELOPMENT IN THE MARINE BIVALVE *HIATELLA FLACCIDA*[1)]

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Gonads of the marine bivalve *Hiatella flaccida* mature in winter. Oocytes possessing the germinal vesicle (GV-oocytes or prophase-I oocytes) were taken directly from the ovary. GV-oocytes underwent germinal vesicle breakdown (GVBD) in seawater containing ionomycin, A23187, serotonin or NH_4Cl. When GV-oocytes were exposed to 5 μM A23187 for 5 min or 5 mM NH_4Cl (pH 9.3) for 20 min and returned to agent-free seawater, they were arrested again at metaphase-I after GVBD. The oocytes arrested at metaphase-I (metaphase-I oocytes) resumed meiosis after fertilization, and developed to normal larvae.

Introduction

The marine bivalve, *Hiatella flaccida*, abounds in Mutsu Bay, and mature adults can be easily collected in winter. In the present study, we used *Hiatella* as a new material for embryological investigations. First, we examined the procedures of artificial induction of meiotic maturation and the outline of early development. Oocytes possessing the germinal vesicle (prophase-I oocytes or GV-oocytes) were obtainable directly from the ovary. Several agents known to induce meiosis resumption in other species were used for the induction of germinal vesicle breakdown (GVBD). We succeeded in inducing GVBD and obtaining metaphase-I oocytes in *Hiatella*. Metaphase-I oocytes accepted sperm and began to develop when they were inseminated.

In this paper, the artificial induction of meiotic maturation and the outline of early development in *Hiatella* are described.

Materials and Methods

Hiatella flaccida were collected in Mutsu Bay, Aomori, from November to December, 1992 and kept in running seawater. Gametes were obtained directly from the gonad with a forceps in seawater. Oocytes were washed several times with seawater and kept at room temperature (19°C). Sperm suspension was kept at 4°C

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until use.

To examine artificial induction of GVBD, GV-oocytes were incubated in seawater containing the following chemical agents for 30 min at 19°C: ionomycin (1 μM), A23187 (5 μM), serotonin (10 μM), isotonic KCl (20%), and NH_4Cl (5 mM). NH_4Cl was buffered with 10 mM tris (hydroxymethyl)-aminomethane (Tris) and adjusted to pH 7.8-9.6 with HCl or NaOH. As stock solutions, 0.2 mM ionomycin and 1 mM A23187 were dissolved in the mixture of dimethyl sulfoxide and ethanol (DMSO-ethanol, 1 : 3). 10 mM serotonin, 0.52 M KCl , 0.4 M NH_4Cl, and 1 M Tris were dissolved in distilled water. The percentage of GVBD was determined by counting at least 200 oocytes per sample. Additional details are given in the text.

Results

Ovarian Oocytes

The shell length of adults of *Hiatella* was about 10-30 mm. The sexes were easily distinguishable because the different color of the gonad was visible through the shell. The ovary is orange, and the testis is white. Oocytes could be obtained by dissecting the ovary in seawater. They were about 60 μm in diameter, and had the germinal vesicle (Fig. 1-a). Spontaneous GVBD occurred in variable percentages from batch to batch (0-30%, data not shown).

Induction of GVBD and Fertility of the Oocytes

GV-oocytes were inseminated, but they did not show any sign of fertilization. Accordingly, induction of GVBD was examined using 1 μM ionomycin, 5 μM A23187, 10 μM serotonin, 20% v/v isotonic KCl (0.52 M), and 5 mM NH_4Cl (Table 1). GV-oocytes were incubated in seawater containing these chemical agents for 30 min. The oocytes underwent GVBD in seawater containing ionomycin, A23187 or

Table 1.
Artificial induction of GVBD

Agent	% GVBD*
1 μM ionomycin	100
5 μM A23187	99.8
10 μM serotonin	99.4
20% KCl	7.6
5 mM NH_4Cl (buffered with 10 mM Tris, pH 9.3)**	100
0.5% DMSO-ethanol	4.5
0.5% distilled water	4.5

GV-oocytes were incubated in seawater containing agents for 30 min.
* Mean results of three experiments with three batches of oocytes.
** External pH dependence is shown in Table 2.

serotonin, and the rates of GVBD were 100%, 99.8%, or 99.4%, respectively (Table 1). Though NH_4Cl was also effective at inducing GVBD, the ability to induce GVBD depended upon external pH (Table 2). When external pH was adjusted at 9.3 or 9.6, oocytes underwent GVBD at rates of 99.8% or 99.8%, respectively (Table 2). GVBD was, however, induced to only a small extent when pH was adjusted at

Table 2.
Effect of external pH on GVBD with NH_4Cl

pH	% GVBD*
7.8	12.2
8.1	11.7
8.4	13.1
8.7	33.2
9.0	78.6
9.3	99.8
9.6	99.8

GV-oocytes were incubated in seawater containing 5 mM NH_4Cl and 10 mM Tris (pH was adjusted with NaOH or HCl) for 30 min.
* Mean results of three experiments with three batches of oocytes.

Table 3.
Time table of early development of *Hiatella* (19°C)

Stage	Time after insemination	Remarks
Unfertilized metaphase-I oocyte		Vitelline membrane appears to increase in thickness.
1st polar body formation	20 min	
2nd polar body formation	40 min	
2-cell stage	70 min	Polar lobe does not appear during cleavage.
"3-cell" stage	110 min	Large blastomere cleaves faster than small blastomere.
4-cell stage	120 min	Cleavage of the small blastomere is delayed.
"5-cell" stage	140 min	Large blastomere cleaves faster than small blastomers.
8-cell stage	160 min	Cleavages of small blastomeres are delayed.
trochophore	8 h	
	16 h	Apical tuft is clearly visible.
	20 h	
D-shaped larva	24 h	
	40 h	Apical tuft disappears.

8.7 or below (Table 2). KCl had no effect on induction of GVBD (Table 1). Metaphase-I oocytes obtained by exposure to the agents mentioned above were subsequently fertilized by insemination and began to develop.

We frequently observed the extrusion of polar bodies after GVBD induced by exposing GV-oocytes to ionomycin, A23187, serotonin and NH_4Cl for 30 min or longer. These oocytes did not develop when they were inseminated.

To obtain fertilizable metaphase-I oocytes, therefore, GV- oocytes were exposed to either 5 μM A23187 for 5 min or 5 mM NH_4Cl (buffered with 10 mM Tris, pH 9.3) for 20 min, and then washed three times with seawater. Following these treatments, oocytes were arrested at metaphase-I after GVBD (Fig. 1-b).

Outline of Early Development

The vitelline membrane of unfertilized oocytes (metaphase-I oocytes) slightly increased in thickness (Fig. 1-b). These oocytes were then inseminated to observe early development. The developmental times at 19°C are shown in Table 3 and Fig. 1. The first polar body was extruded about 20 min after insemination (Fig. 1-c) and the second polar body appeared about 40 min after insemination (Fig. 1-d).

The first cleavage was observed about 70 min after insemination and the oocyte

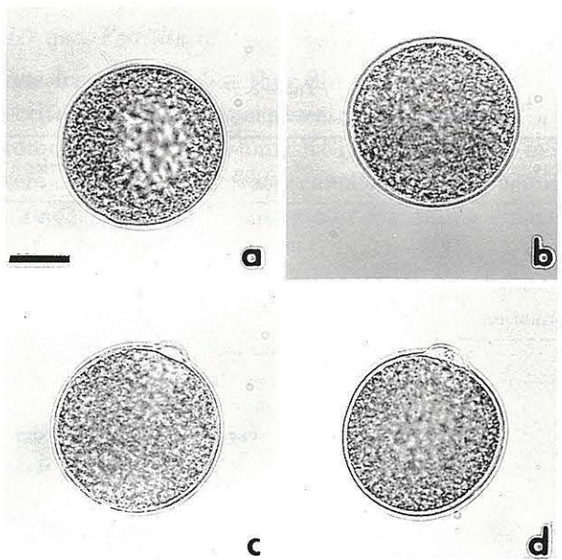

Fig. 1. The process of meiosis and early development in *Hiatella* at 19°C.
(a) GV-oocyte taken from the ovary. (b) Metaphase-I oocyte obtained by treatment with A23187 or NH_4Cl (pH 9.3). Vitelline membrane slightly increases in thickness. (c) Extrusion of 1st polar body (20 min after insemination). (d) Extrusion of 2nd polar body (40 min after insemination). Scale bar = 20 μm.

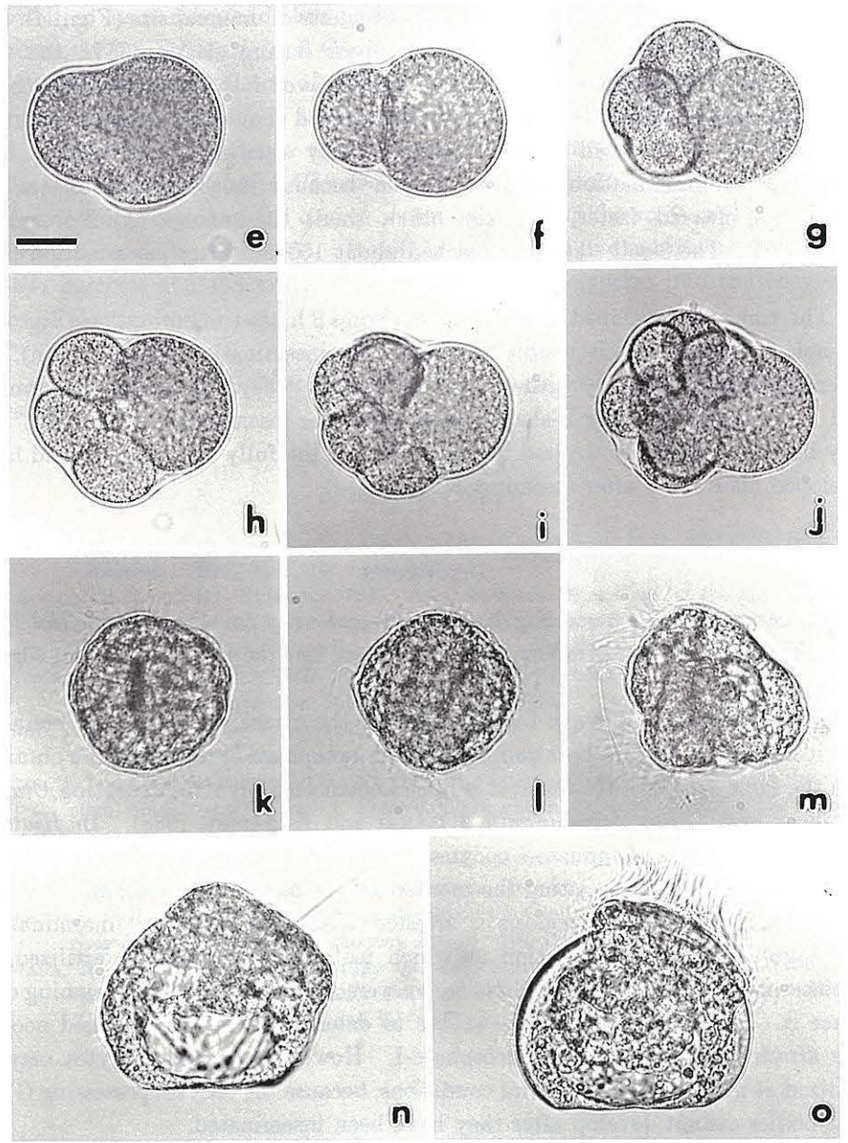

Fig. 1. (continuation).
(e) First cleavage. Polar lobe does not appear during cleavage. (f) 2-cell stage (70 min after insemination). (g) "3-cell" stage. CD-blastomere cleaves faster than AB-blastomere. (h) 4-cell stage (120 min after insemination). (i) "5-cell" stage. D-blastomere cleaves faster than other blastomeres. (j) 8-cell stage (160 min after insemination). (k) Early trochophore (about 8 h after insemination). (l) Mid trochophore (about 16 h after insemination). Apical tuft is visible. (m) Late trochophore (about 20 h after insemination). Long apical tuft can be seen. (n) Early D-shaped larva (about 24 h after insemination). (o) D-shaped larva (about 40 h after insemination). Apical tuft has disappeared. Scale bar = 20 μm.

reached the 2-cell stage, consisting of two blastomeres of unequal size (Fig. 1-f). As shown in Fig. 1-e, a polar lobe did not appear during cleavage. In the next cleavage, the large blastomere (CD-blastomere) cleaved faster than the smaller one (AB-blastomere); the 4-cell stage therefore developed about 120 min after insemination (Fig. 1-h) via a "3-cell" stage (Fig. 1-g). They were in a "5-cell" stage about 140 min after insemination (Fig. 1-i), again because the large blastomere (D-blastomere) cleaved faster than the other, small blastomeres (A-, B-, and C-blastomere). The 8-cell stage was reached about 160 min after insemination (Fig. 1-j).

The embryos developed to early trochophores 8 h after insemination (Fig. 1-k). The apical tuft was clearly visible from 16 h after insemination (Fig. 1-l, m, n), and the embryos swam actively with ciliary movement. They then started to form the shell, and formed the early D-shaped larva 24 h after insemination (Fig. 1-n). The body became completely covered by the shell, and the fully formed D-shaped larva developed about 40 h after insemination (Fig. 1-o).

Discussion

As shown in Table 3 and Fig. 1, the outline of early development in *Hiatella* is almost the same as that of other marine bivalves, but the polar lobe is not formed during cleavage.

As an experimental model for investigating the mechanisms of meiosis reinitiation, it will be useful that both prophase-1 and metaphase-I oocytes can be obtained from the same species. However, it is little known such bivalves excepting *Crassostrea gigas* and *Tapes philippinarum* (Osanai and Kuraishi, 1988). In *Hiatella*, both prophase-I and metaphase-I oocytes can be obtained, so this bivalve will be a useful material for investigating the mechanisms of meiosis reinitiation.

In molluscan oocytes, meiosis is arrested after spawning until insemination. The stages of meiotic maturation at which molluscan oocytes are fertilized are prophase-I or metaphase-I. In *Hiatella*, we were not able to observe spawning or to induce it. Consequently we were unable to determine whether spawned oocytes were arrested at prophase-I or metaphase-I. However, *Hiatella* oocytes may be fertilized at metaphase-I in natural conditions, because the oocytes possessing GV or polar bodies cannot develop after they have been inseminated.

Although it has been reported that the vitelline membrane appears to increase somewhat in thickness after fertilization or artificial activation in *Spisula solidissima* and *Mactra veneriformis* (Allen, 1953; Rebhun, 1962; Sawada, 1964; Longo and Anderson, 1970), there is no report of this occurring before fertilization in marine bivalves. In *Hiatella*, slight thickening of the vitelline membrane occurs before fertilization (Fig. 1-b). Therefore, *Hiatella* sperm may penetrate this vitelline membrane during fertilization. The sperm undergoes the acrosome reaction on

the vitelline membrane, and then protrudes the acrosomal process (Hylander and Summers, 1977; Kyozuka and Osanai, 1985). But it has not been firmly established whether the actual site of gamete fusion in molluscs is at the microvillar or intervillar region of the egg surface (Longo, 1983). Hylander and Summers (1977) provided the evidence that gamete fusion occurs between the acrosomal process and the microvillus in *Chama macerophylla*. *Crassostrea* sperm, however, fuse at the intervillar region of the oocyte cortex by inserting the acrosomal process into it (Kyozuka and Osanai, 1985). It will be interesting to examine the structure of *Hiatella* gametes and the process of penetration of the sperm into the oocyte by means of electron microscopy.

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